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Use of a Novel Platelet Function Analyzer (PFA-100™) With High Sensitivity to Disturbances in von Willebrand Factor to Screen for von Willebrand's Disease and Other Disorders

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The PFA-100™ is a new platelet function analyzer which uses whole blood and high shear stress blood flow to simulate primary hemostasis and assess platelet function. A small volume of blood is introduced into a disposable cartridge, and forced through a capillary tube. Platelet adhesion and aggregation is then initiated following exposure to either collagen/ADP [C/ADP] or collagen/epinephrine [C/Epi] coated membranes. Movement of blood through the capillary, and its subsequent occlusion is monitored and yields the measured endpoint (closure time [CT] in seconds). Using two approaches, we assessed the sensitivity of this system to disturbances in the function of von Willebrand Factor (VWF). Firstly, we assessed the ability of the PFA-100™ to detect the presence of von Willebrands Disease (VWD). Using normal individuals (*N* = 18), CTs (in seconds; mean [range = mean ± 2SD]) were (i) C/ADP, 95 [66–124], (ii) C/Epi, 128 [98–158]. A panel of 47 patients undergoing evaluation for clinical hemostatic defects inclusive of VWD were also evaluated. All samples from patients confirmed to have VWD following specific VWF studies [*N* = 9; 3 × Type 1, 1 × Type 3, 1 × Type 2A, 4 × Type 2B] gave prolonged CTs (≥ 200 s) for both C/ADP and C/Epi membranes; in contrast, all patients yielding normal CT values were found to yield normal VWF results (i.e., were found not to suffer from VWD). Patients with hemophilia (1 × hemophilia A, 1 × hemophilia C) gave normal PFA-100™ CT, while those with clinical thrombocytopaenia (*N* = 3) gave prolonged PFA-100™ CT. A number of other patient samples also gave abnormal CT values which in some cases could be linked to recent aspirin consumption. In the second evaluation process, and using normal blood, we have assessed the ability of various antibodies to influence the CT. Of the monoclonal antibody panel tested [*N* = 20], only a proportion of those against VWF [6/10] or gp1b/IX [CD42; 2/5] were found to be inhibitory (i.e., prolonged the CT). Data using polyclonal antibodies (against platelets, VWF, fibrinogen and fibronectin) is more complex but largely confirms the sensitivity of the system to VWF. On the basis of these results, we conclude that the PFA-100™ is highly sensitive to disturbances in VWF and to the presence of VWD and may thus provide a valuable screening test for VWD in certain specific circumstances (i.e., acute need conditions or remote testing sites; normal CT result generally effective as negative predictor, i.e. not severe VWD). However, since abnormal CT values were obtained in clinical situations other than evident VWD, the PFA-100™ cannot be used as a specific diagnostic tool to establish the presence of VWD. Thus, any abnormal PFA-100™ CT result should be thoroughly evaluated by follow-up specific testing to establish the true clinical disorder affecting the individual under investigation, inclusive of appropriate VWF assays if VWD is clinically suspected. Am. J. Hematol. 62:165–174, 1999.

Key words: von Willebrand factor; von Willebrand's disease; laboratory assessment; screening; diagnosis

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INTRODUCTION

von Willebrand's disease (VWD) is now recognized to be the most common inherited bleeding disorder [1]. Patients suffering VWD have defects in, or have reduced levels of, von Willebrand factor (VWF), an adhesive plasma protein with two primary hemostatic roles (permits adhesion of platelets to sites of vascular damage and stabilizes factor VIII coagulation function) [1–4]. VWD is a heterogeneous disorder, and patients are typed according to pathophysiology with classification protocols involving both clinical observations and laboratory analysis [1–6]. In order to appropriately diagnose a patient with VWD, a panel of laboratory assays is typically required, not necessarily restricted to an assessment of VWF [7]. This is due to both the presence of VWD heterogeneity and because of the diagnostic limitations of any given laboratory assay. Thus, no single test procedure for VWF (including VWF:Multimer analysis, VWF:Antigen (VWF:Ag), VWF:Ristocetin Cofactor (VWF:RCof), and VWF:Collagen Binding Activity assay (VWF:CBA)) is sufficiently robust to permit detection of all VWD variants [7].

In addition, there is a significant problem associated with the speciality in testing required, as well as the time frame required for the testing process. Thus, most of the current available tests either require a high degree of speciality because of technical complexity, or are time consuming and cumbersome to perform. The laboratory confirmation for the presence, or proper clinical diagnosis, of VWD may take a week or so to complete. However, a quick and easy VWD-screening procedure would be of considerable value, given certain clinical scenarios and because the presence of VWD can significantly affect the therapeutic management of affected patients. Examples of acceptable scenarios where VWD-screening may be warranted in order to aid subsequent clinical management would include remote testing sites and acute need situations (these would include: patients undergoing imminent surgery, particularly where the patient [bleeding] history is unclear or when the clinician may not be a hematology specialist, unexpected post surgical bleeding, and acute spontaneous bleeding episodes [e.g., severe nosebleed] presenting in the emergency ward in the absence of a definitive previous diagnosis). In the current report, we show that a newly developed instrument (the PFA-100™) may fulfil this urgent VWD-screening role, particularly when used as a "negative" prediction tool.

Abbreviations

C/ADP	collagen/ADP (cartridge)
C/Epi	collagen/epinephrine (cartridge)
CT(s)	closure time(s)
PFA	platelet function analyzer
PFS	platelet function studies
RIPA	ristocetin-induced platelet aggregation
SBT	skin bleeding time(s)
VWD	von Willebrands disease
VWF	von Willebrand factor
VWF:Ag	von Willebrand factor antigen (assay)
VWF:CBA	collagen binding activity [assay] for VWF
VWF:RCof	ristocetin cofactor assay for VWF

MATERIALS AND METHODS

Blood Samples: Patients and Normal Controls

This report deals with testing of blood obtained from either normal individuals [$n = 18$] or from patients undergoing evaluation for VWD or other clinical hemostatic disorders [$n = 47$]. All subjects were informed that they were undergoing laboratory testing and gave appropriate consent. All blood was collected into standard sodium citrate tubes (0.105 M; 9 parts blood to 1 part sodium citrate; Vacutainer, Becton Dickinson, UK). On occasion, blood was collected through both the vacuum process (i.e., typical Vacutainer collection process) as well as through a syringe system, in order to compare laboratory results (i.e., test for any effect of vacutainer collection). Samples from normal subjects (i.e., individuals devoid of any hemostatic defect) were used to establish normal ("reference") ranges for the PFA-100™ (see below), as well as for control purposes and in the antibody inhibition studies (see below). From the patient sample group, nine individuals with well defined VWD could be identified (3 × Type 1, 1 × Type 3, 1 × Type 2A, 4 × Type 2B) as well as a further three individuals with clinical thrombocytopaenia, one individual with hemophilia A, and one individual with hemophilia C.

Laboratory Tests

These included (i) assessment of platelet function using the PFA-100™ (see below), and occasionally also using standard methodologies as previously described [8]; (ii) workups for VWD (including assessment of Factor VIII:coagulant (FVIII:C), VWF:antigen (VWF:Ag), VWF:ristocetin cofactor (VWF:RCof), VWF:collagen binding activity assay (VWF:CBA), and ristocetin-induced platelet aggregation (RIPA) and/or VWF:multimer analysis where relevant [see refs 9–11 for previously published methodologies]; (iii) routine coagulation workups (prothrombin time [PT] and activated partial thromboplastin time [APTT]) using the MDA-180 coagulation analyzer [12]; and (iv) other tests of specific coagulation factors, as required, using routine established laboratory procedures [12]. Some patients with VWD were also evaluated with DNA techniques [13]. Blood counts (including platelet counts) were performed using a Technicon-H2 analyzer (Bayer, Sydney, Australia).

PFA-100™ Test System

The test principles and characteristics of this instrument have been previously outlined [14–16]. In brief, the PFA-100™ uses blood flow through a capillary device to mimic high shear stress conditions that occur in-vivo (i.e., "simulates" primary hemostasis). The instrument gives a single end-point reading (when blood flow through the device ceases [i.e., capillary device is occluded] as a result of platelet aggregation in response to exposure to platelet agonists coated onto a membrane in a disposable cartridge device; this end-point is called the "closure time" = CT). There are currently two cartridge types available: both utilize a membrane coated with collagen (fibrillar type I equine tendon); in addition, one cartridge membrane type is additionally coated with epinephrine (10 μg = C/Epi cartridge) and the other cartridge membrane type ADP (50 μg = C/ADP cartridge). The theoretical maximum CT provided by the PFA-100™ is 300 s. In practice, however, this value is rarely achieved, and the instrument tends to report values as >250–299 s as its maximum CT value. Thus, for the sake of this report, CT values >250 s are considered as maximal.

Antibody Studies

These involved the use of both polyclonal antibodies (rabbit-anti-mouse immunoglobulin, rabbit-anti-VWF, rabbit-anti-fibronectin, rabbit-anti-fibrinogen, and rabbit-anti-thrombocyte, all from Dakopatts, Sydney, Australia) and monoclonal antibodies (MAB). For the latter, a range of specificities was included, although the primary focus was antibodies to either VWF or to platelets (and primarily to gpIb/IX [CD42]). Many of the antibodies utilized have previously been published, as have their production and functional characterization [17–28; see also Results and Discussion). In total, 20 MAB were tested in this series of experiments, including 10 MAB to VWF, and 5 MAB to CD42. All were in house derived, and purified using standard procedures to a concentration of 1 mg/ml. In brief, MAB or polyclonal antibodies were incubated at various concentrations, ranging from 0 to 100 μg/ml using the equivalent volume (i.e., 0–100 μl/ml) in whole blood, for 5min prior to that blood being introduced into the PFA-100™. Appropriate controls were included in all assays, including irrelevant antibodies utilized at equivalent concentrations, as well as isotonic buffer used at an equivalent volume but without any antibody added. All antibodies were tested at least twice using both cartridge systems at the range of concentrations noted above, or until the maximal CT was obtained (i.e., >250s). For further details, please refer to Results and Discussion. For the purpose of this report, CT values >250 s are considered to be the maximal reportable CT (=100% inhibition when achieved in antibody inhibition assays).

Statistical Analysis

Mean, SD, and reference ranges have either been previously established (i.e., for existing test methodologies [7–12]) or else were determined for all new test parameters using standard procedures and Graphpad PRISM software (version 2.0 for PowerMac; Graphpad Software, USA). The normal range was defined as the mean ± 2SD of the healthy volunteer group. Correlation analysis using linear regression was also undertaken where relevant, as was evaluation of specificity and sensitivity.

RESULTS

Normal Ranges for PFA-100™

Using blood from 18 normal individuals, the closure time (CT) normal reference ranges were found to be (sec; mean [range = mean ± 2SD]): (i) C/ADP cartridge: 95 [66–124], (ii) C/Epi cartridge: 128 [98–158]. These calculated ranges were also reflective of the range of individual data points as evidenced by the actual minimum and maximum values obtained (min, max: C/ADP = 70, 124; C/Epi = 100, 152), as well as those ranges indicated with the manufactured product insert (sec; mean [range = mean ± 2SD]): (i) C/ADP cartridge: 92 [71–118], (ii) C/Epi cartridge: 132 [94–193]. There was no observable difference between CT values obtained from samples collected into Vacutainer tubes via vacuum versus non-vacuum syringe collection (data not shown), and good reproducibility was generally evident when tests were conducted in duplicate (data not shown).

PFA-100™ CT Using Patient Samples

Individual CT values obtained from normal volunteers are further identified in Fig. 1, in comparison to data obtained using samples from individuals undergoing evaluation for VWD and for other potential hemostatic defects ($n = 47$). From this patient group, nine individuals with well defined VWD could be identified (3 × Type 1, 1 × Type 3, 1 × Type 2A, 4 × Type 2B); all these individuals gave extended (i.e., abnormal) CT values (both C/ADP and C/Epi; see Fig. 1). In addition, a further three individuals found to have clinical thrombocytopaenia were also found to give prolonged (i.e., abnormal) CT times (both C/ADP and C/Epi; see Fig. 1). Of the remaining 35 patient samples, eight gave prolonged C/Epi CT but normal C/ADP CT, and many of these individuals were subsequently found to have taken aspirin medication within the previous week. Three individuals gave prolonged C/Epi and C/ADP CTs which could not be

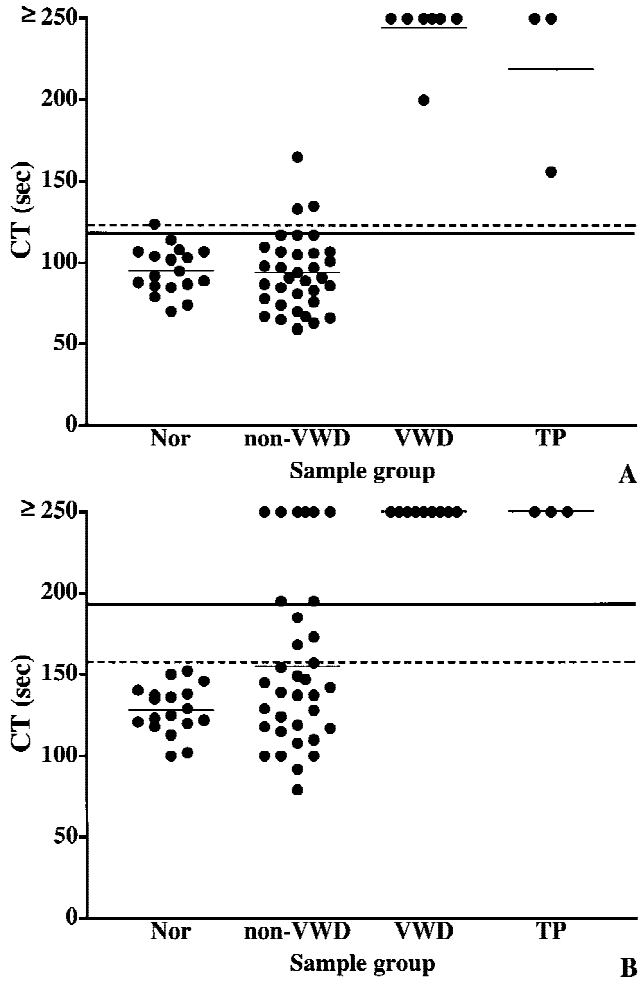

FIG. 1. Scatterplot of individual data points for PFA-100™ CT (s; *y* axis; set to maximum of 250 s) for normal individuals ("Nor" group), patients undergoing evaluation for hemostatic defects including VWD and subsequently shown not to suffer from VWD ("non-VWD" group), patients shown to suffer from VWD ("VWD" group), and patients undergoing evaluation for hemostatic defects shown to suffer clinical thrombocytopaenia ("TP" group). (A) C/ADP cartridge; (B) C/Epi cartridge. Small horizontal line within each group indicates group set's mean value. Long horizontal lines indicate upper limit of normal reference range (dashed line = in house data; unbroken line = product insert data).

explained by follow-up testing, although drug-related effects could not be discounted, and one individual's sample failed to provide CTs (PFA-100™ instrument terminated test due to presence of small clots detected in whole blood sample). All remaining patients ($n = 23$) gave normal CT values (for both C/ADP and C/Epi; see Fig. 1). All of these patients were found to give normal laboratory VWF results, and were subsequently confirmed not to have VWD (i.e., form a normal CT/non-VWD patient group). Two of these individuals, however, were shown to have hemophilia (1 × hemophilia A, Factor VIII = 8%; 1 × hemophilia C, Factor XI = 37%).

In summary, then, prolonged PFA-100™ CT values were obtained in all patients with demonstrated VWD (both C/ADP and C/Epi CT results prolonged), as well as in some patients where VWD could be discounted (these included patients subsequently found to have clinical thrombocytopaenia [both C/ADP and C/Epi CT results prolonged], patients who had recently taken aspirin [C/Epi cartridge only gave prolonged CT], and a few patients where a definitive diagnosis could not be made). Accordingly, for the patient group evaluated in this report, sensitivity of the PFA-100™ for VWD was 100% (i.e., 9/9 VWD patients gave prolonged CTs; both for C/ADP and C/Epi). However, specificity for VWD was only 83.8% (C/ADP) and 62.2% (C/Epi). In order to further correlate laboratory findings, correlation analysis was also undertaken. We found that significant correlation was evident between CT results (both C/ADP and C/Epi) and the test values obtained with any VWF assay and with platelet count, as summarized in Table I.

Antibody Inhibition Studies

Addition of buffer without added antibody, or addition of any irrelevant (i.e., negative control) MAB (e.g., WM-25, WM-15, and WM-47) to normal blood, had little effect on the PFA-100™ CT using either the C/ADP cartridge or the C/Epi cartridge (Fig. 2; all data summarized in Table II), even when using up to 10% of added material by volume (i.e., = 100 μl material/ml whole blood). Of the test MAB tried, only some of those against VWF, or against gpIb/IX (CD42), prolonged the CT of normal blood (see Fig. 3; only C/ADP cartridge data shown; all data summarized in Table II). Of the polyclonal antibodies tested, essentially only that against VWF, or platelets, consistently prolonged the CT of normal blood (see Fig. 4, C/ADP cartridge data only shown; all data summarized in Table II).

DISCUSSION

Limitations in Current VWD Testing Practice

The appropriate diagnosis of VWD requires specialist analysis that does not lend itself well to acute testing conditions. Typically, a panel of test processes are required because of VWD heterogeneity and the individual diagnostic limitations of the available assays [6,7]. Most available VWF assays do not permit for rapid results, and individually would only capture a proportion of VWD patients (i.e., VWF assay test processes are complimentary rather than "stand-alone"). For example, while potentially able to provide fast results, the two available "automated" VWF assays (the STAGO Latex immunoassay (LIA) test, and the bioMerieux VIDAS system) are nonfunctional assays (i.e., detect VWF:Ag) and these would be expected to miss some Type 2 VWD variants (i.e., some of these patients have normal VWF antigen

TABLE I. Linear Regression Analysis: PFA-100™ CT Versus VWF Parameters and Platelet Count

		(i) C/ADP cartridge, CT vs			(ii) C/Epi cartridge, CT vs		
Test parameter	*N*	*R*	*P* value	Comment[e]	*R*	*P* value	Comment
C/ADP CT	64[a]	1.000		Perfect line	0.7874	<0.0001	Significant
C/ADP CT	53[b]	1.000		Perfect line	0.9479	<0.0001	Significant
C/ADP CT	50[c]	1.000		Perfect line	0.9641	<0.0001	Significant
vWF:Ag	40[c]	0.6037	<0.0001	Significant	0.5987	<0.0001	Significant
vWF:RCof	16[c]	0.7440	0.0010	Significant	0.6902	0.0031	Significant
vWF:CBA	40[c]	0.7218	<0.0001	Significant	0.6964	<0.0001	Significant
Ag to CBA ratio	40[c]	0.4387	0.0046	Significant	0.3847	0.0142	Significant
FVIII:C	24[c]	0.3559	0.0878	Not significant	0.3901	0.0594	Not significant
C/ADP CT	44[d]	1.000		Perfect line	0.8835	<0.0001	Significant
Platelet count	30[d]	0.5667	0.0011	Significant	0.6148	0.0003	Significant

[a]All patient [$N = 47$] and normal [$N = 18$] data included (NB: one patient sample failed to give CT values).
[b]Using data for "true positives" (i.e., abnormal CT values with VWD and TP samples) and "true negatives" (i.e., normal CT values from normal samples and non-VWD/non-TP patient samples).
[c]Using data for "true positives" (VWD samples only) and "true negatives" (i.e., normal CT values from normal samples and non-VWD/non-TP patient samples).
[d]Using data for "true positives" (TP samples only) and "true negatives" (i.e., normal CT values from normal samples and non-VWD/non-TP patient samples.)
[e]Significance assigned at level $P < 0.05$.

levels, but the VWF is "dysfunctional"). Additionally, a low VWF:Ag may be determined and suggest the presence of VWD, but this alone will provide no information on VWD subtype. These limitations create a substantial problem since appropriate clinical (i.e., therapeutic) management is dependent on the appropriate diagnosis and subclassification of the patient under investigation. In addition, the LIA test is known to give falsely high values of VWF if rheumatoid factor is present.

The other available VWF assays (e.g., VWF:Ag and VWF:CBA by ELISA assay, VWF:RCof by platelet agglutination, and VWF:multimer analysis) are best performed as batch assays because of the testing time involved [7]. Although the VWF:RCof assay is a "functional" assay and can theoretically be performed in a "stat" mode, this assay suffers considerable technical problems, including considerable assay variability, which limits its overall usefulness [7,9,11,29]. Other options, such as performance of skin bleeding times (SBT) or conventional platelet function studies (PFS), are limiting, are time consuming, have poor reproducibility, and have poor sensitivity and specificity for VWD [30–32]. The latter test typically requires considerable expertise, and considerable sample handling and processing (which itself potentially leads to testing artifacts). It is best used selectively in the VWD diagnostic process (i.e., as a ristocetin-induced platelet agglutination [RIPA] procedure), where it is of some value in subtyping Type 2 VWD (i.e., to help distinguish Type 2A from Type 2B VWD [30,31]).

However, in our experience, there are valid occasions where a quick screening process may be required, and can be justified, in order to establish the likelihood or otherwise of VWD, and therefore permit optimization of

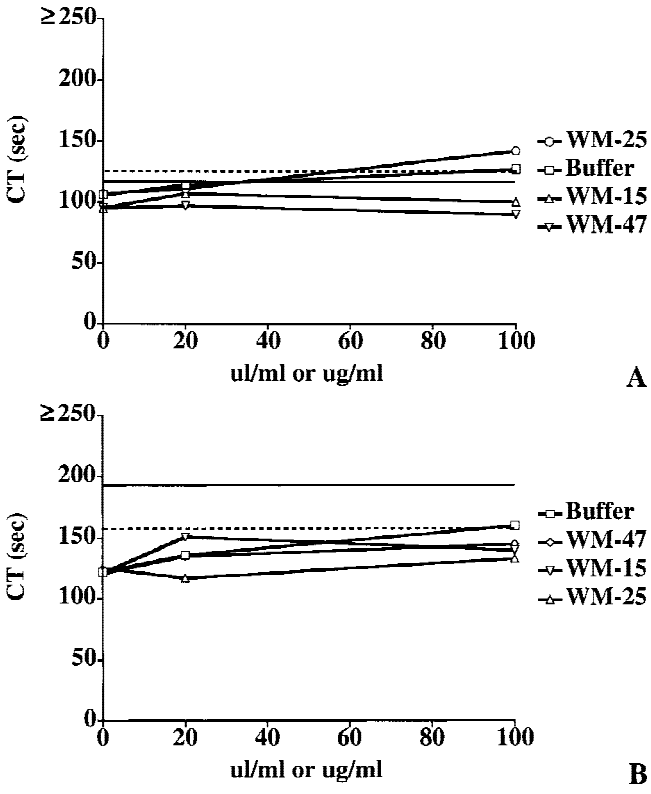

FIG. 2. Effect of addition of various ("negative control") MAB, or buffer used without MAB, on PFA-100™ CT (s; *y* axis; set to maximum of 250 s). MAB tested at various concentrations (μg/ml whole blood final, and representing equivalent volume of added buffer in μl/ml whole blood final; buffer without MAB used at equivalent range of volumes; *x* axis). Sample experimental data shown. (A) C/ADP cartridge; (B) C/Epi cartridge. Long horizontal lines indicate upper limit of normal reference range (dashed line = in house data; unbroken line = product insert data).

TABLE II. Antibody Inhibition Study Data Summary*

Antibody details	Reference	CD/antibody group	C/ADP CT		C/Epi CT	
			% inhibition[a]	grade[b]	% inhibition[a]	grade[b]
1. Monoclonal antibodies						
(i) Negative controls						
WM-25	17,18	CD1b (neg. cont.)	3.0	−	−5.8	−
WM-15	19,20	CD13 (neg. cont.)	−2.0	−	−3.8	−
WM-47	20	CD13 (neg. cont.)	1.1	−	8.7	−
(ii) Anti-platelet MAB						
WM-59	21	CD31	−5.1	−	−0.3	−
AK-6	22,23	CD62P	−4.8	−	−1.7	−
AK-1	24–26	CD42	20.9	+	23.4	+
AK-2	25,26	CD42b	100	++++	100	++++
AK-3	24–26	CD42	22.3	+	28	+
FMC-25	24–26	CD42a	11.3	−	11.8	−
WM-23	24–26	CD42	31.7	++	53.2	++
(iii) Anti-vWF MAB						
2C9	27,28	Anti-vWF	89.6	+++	100	++++
5D2	28	Anti-vWF	100	++++	100	++++
6G1	28	Anti-vWF	100	++++	100	++++
CR1	28	Anti-vWF	100	++++	100	++++
CR2	28	Anti-vWF	100	++++	100	++++
CR3	28	Anti-vWF	5.5	−	2.0	−
CR4	28	Anti-vWF	−7.5	−	5.4	−
CR7	28	Anti-vWF	−1.0	−	−8.7	−
CR11	28	Anti-vWF	6.2	−	18.2	−
CR15	28	Anti-vWF	35.0	+	65.7	+++
2. Hetero-antisera: Rabbit-anti						
Mouse immunoglobulin		(Neg. cont.)	−5.9	−	6.7	−
vWF		Anti-vWF	100	++++	100	++++
Fibronectin		Anti-fibronectin	3.5	−	48.3	++
Fibrinogen		Anti-fibrinogen	13.3	−	7.6	−
Thrombocyte		Anti-thrombocyte	100	++++	100	++++

*Data on inhibition for negative controls ("neg. cont.") is relative to the use of an equivalent volume of buffer containing no antibody; subsequent data using test antibodies is relative to negative controls.

[a]% Inhibition at highest tested antibody concentration (i.e., that yielding greatest inhibition; typically 20 or 100 μg/ml final); mean of at least 2 experimental points.

[b]Grade of inhibition interpreted from % inhibition data and expressed on an incremental scale, rising in 20% increments: thus − = <20% inhibition; + = 21–40% inhibition; ++ = 41–60% inhibition; +++ = 61–80% inhibition; ++++ = >80% inhibition.

the clinical management of the patients under evaluation. These "acute-need" situations will include imminent surgery, acute post-surgical bleeding complications, spontaneous acute bleeding (e.g., severe nosebleed presenting to emergency ward), and selective instances in remote testing sites where referral to specialist VWF sites may be difficult to arrange. Such occasions may be further complicated by an inability to immediately consult with specialist Hematologists (e.g., remote sites), or an inability to obtain an adequate confirmatory or strong clinical history (e.g., bleeding in operating theater). A provisional "diagnosis" of VWD, or at least the ability to discount VWD in a given patient, would be of some value in the clinical management of these patients.

The PFA-100™ is small, portable, very easy to use, swift, uses disposable cartridges, involves minimal training, minimal blood handling, little technical expertise, and only requires a small sample of whole blood. Although this instrument is described as a "platelet function analyzer", it is quite different to other available (or conventional) instruments. Interestingly, collection of blood through a vacutainer system does not appear to pose a testing problem. As we also clearly show in this report, the PFA-100™ is also very sensitive to disturbances in VWF. This was demonstrated using two separate approaches.

High sensitivity of the PFA-100™ to VWD. In the first study approach, we tested blood samples from a panel of patients undergoing evaluation for hemostatic defects including VWD. From this group, all those shown to have VWD were found to have prolonged PFA-100™ CT values (i.e., PFA-100™ showed 100% sensitivity to VWD in our study). CT results could also be shown to correlate well with every measured VWF parameter or platelet count (Table I). These findings are similar to those recently described by Fressinaud et al. [32]. In this large French VWD study, abnormal PFA-

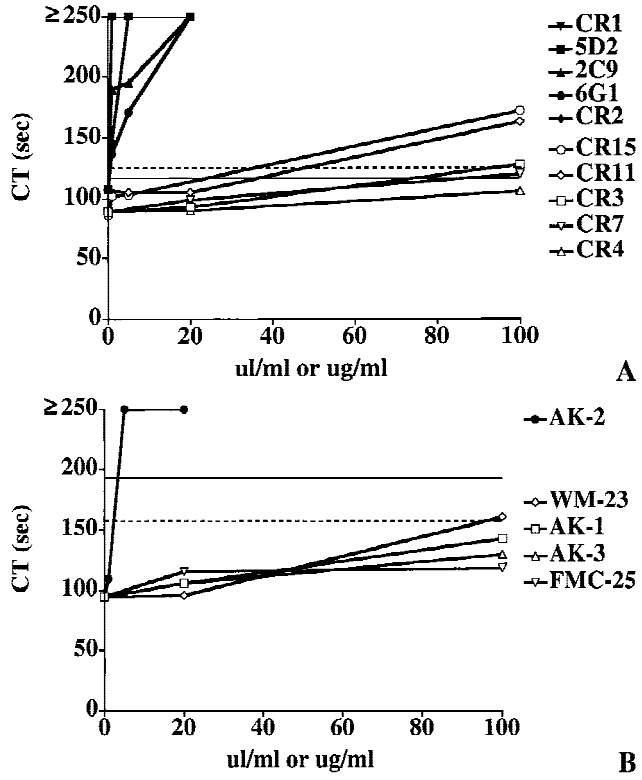

FIG. 3. Effect of addition of various anti-VWF MAB (A) and anti-gpIb/IX (i.e., CD42) MAB (B) on PFA-100™ CT (s; *y* axis; set to maximum of 250 s). MAB and buffer conditions tested as per Fig. 2. Sample experimental data shown for C/ADP cartridge only. Long horizontal lines indicate upper limit of normal reference range (dashed line = in house data; unbroken line = product insert data).

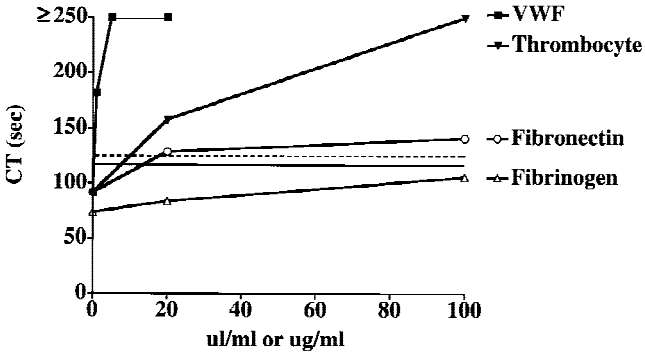

FIG. 4. Effect of addition of various antisera on PFA-100™ CT (s; C/ADP cartridge; *y* axis set to maximum of 250 s). Antisera concentrations and testing conditions as per MAB and buffer conditions (see Fig. 2). Sample experimental data shown. Long horizontal lines indicate upper limit of normal reference range (dashed line = in house data; unbroken line = product insert data).

100™ CT were found in all but four VWD patients (two patients with Type 2N VWD, and two patients with mild Type 1 VWD). The two Type 2N VWD patients would not have been expected to yield abnormal CT; the two Mild Type 1 VWD patients had only marginally low VWF (VWF:RCof and VWF:Ag) values. CT results could be correlated to VWF:RCof values ($R = 0.62$ for C/ADP; $R = 0.56$ for C/Epi), and the PFA-100™ was found to be a superior VWD screening tool to skin bleeding times [32]. Data using the VWF:CBA assay was not obtained in this French study. Although numbers in our study are relatively small, we were interested to observe that correlation between the PFA-100™ CT and VWF:CBA values was quite strong (see Table I). The benefits and strengths of using the VWF:CBA assay have previously been outlined in some detail [7,9–11,29,31,33,34]. The PFA-100™ was also recently shown to be sensitive to Type 3 VWD in another recent study [4/4 patients gave abnormal CT results; 35].

Lack of specificity of the PFA-100™ for VWD. Although most other patients in our study, including two with hemophilia (1 × hemophilia A; 1 × hemophilia C), gave normal closure times in parallel with normal VWF results and normal platelet counts, prolonged CTs were obtained with samples from some other patients. Thus, patients with clinical thrombocytopaenia, or who recently took aspirin, also had prolonged CTs, as did a few patients without evident VWD or thrombocytopaenia. In the French study [32], a group of 12 hemophilia A, and 2 hemophilia B, patients were also evaluated, and these were found to yield normal CT. A small group of patients with various platelet disorders were also tested; thus, two patients with Glanzmann thrombasthenia, three patients with platelet (pseudo-)type "VWD" and four patients with storage pool disease all gave abnormal CT using both the C/ADP and C/Epi cartridges. A further six patients with an "aspirin-like" defect were also described, and these yielded a prolonged CT with the C/Epi cartridge. Aspirin has also been previously shown to yield prolonged CT somewhat "selectively" in the C/Epi cartridge [14]. Another recent study has also described abnormal CT values obtained in a variety of patient disorders including Glanzmann's thrombasthenia, Bernard Soulier syndrome, storage pool disease and Hermansky Pudlak syndrome [35]. Kundu et al. [16] also showed that the CT was dependent on the platelet count and the haematocrit. Thus, abnormal PFA-100™ CTs are not specific for VWD.

Sensitivity of the PFA-100™ to disturbances in VWF/gpIb-IX interaction. In the second study approach, a variety of antibodies were evaluated for their ability to inhibit the PFA-100™ CT. From this panel, only antibodies to VWF (both polyclonal and MAB) or to platelets (polyclonal: rabbit anti-thrombocyte; MAB to CD42) were found to prolong the PFA-100™ CT. At these CT inhibitory concentrations, polyclonal antibodies to Fibrinogen and Fibronectin were largely without effect, as were MAB to other platelet components. Of the

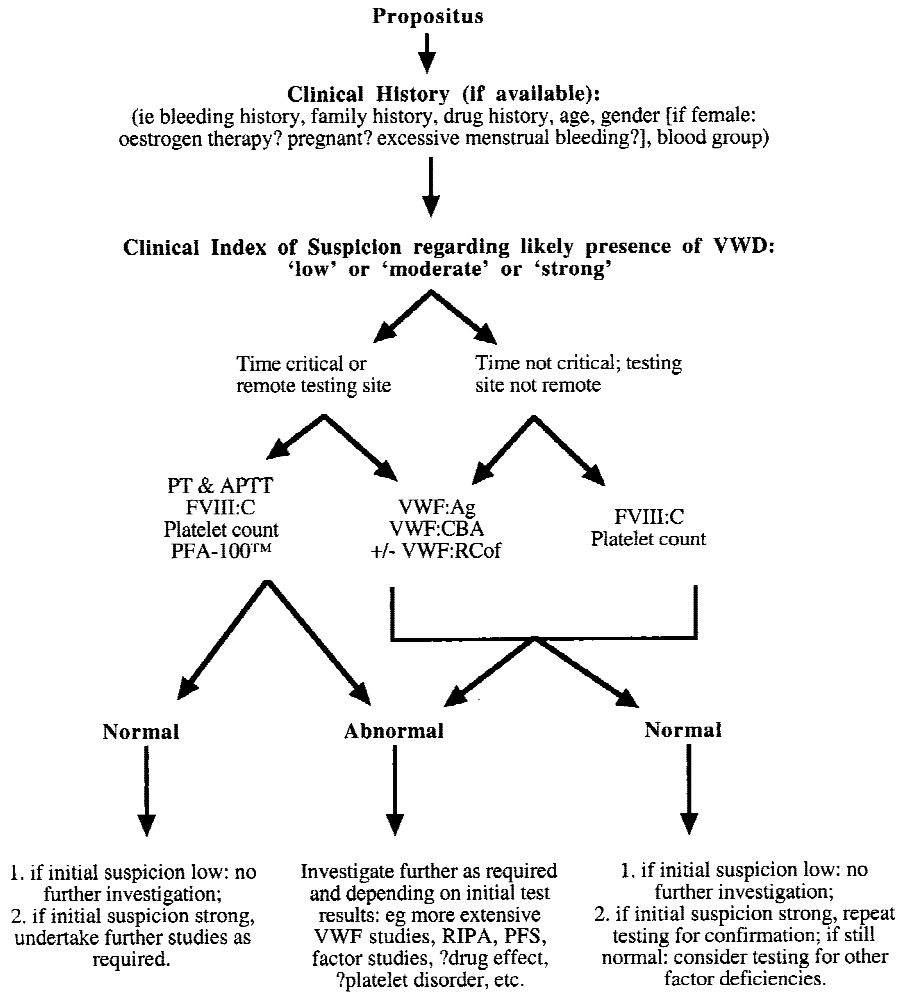

FIG. 5. Algorithm for a recommended approach to the investigation of patients potentially suffering from VWD and incorporating the use of the PFA-100™. Thus, if time is critical, or if testing sites are remote, preliminary performance of routine coagulation tests (PT and APTT), platelet count, FVIII:C and PFA-100™ CT, may be indicated to provisionally assist in the assessment of the likelihood of VWD. If time is not critical, or if testing sites are not remote, then performance of PT, APTT, and PFA-100™ can be omitted if VWD is being specifically investigated, since none of these procedures are diagnostic for VWD. At all times, clinicians should be guided by the propositus's clinical history if available. For further discussion regarding the correct clinical approach, please refer to elsewhere [5,6,30]. This algorithm should be treated as a general guide only; all patients are individuals and should be treated as such.

panel of CD42 MAB tested, AK-2 was the one most capable of CT inhibition. AK-2 has previously been shown to bind to gpIb at the VWF binding site, and is thus capable of inhibiting the binding of VWF to gpIb/IX [25]. The other CD42 MAB tested (AK-1, AK-3, WM-23, FMC-25) are known to bind to distinct sites on gpIb/IX [25] and do not inhibit the binding of VWF to gpIb/IX [25]; similarly, these MAB had only marginal or no effect on the PFA-100™ CT (Table II). Of the panel of VWF MAB tested, over half were inhibitory to PFA-100™ CT. The anti-VWF MAB 2C9 has previously been characterized [27] and only partially inhibits the binding of VWF to gpIb/IX [25]. Although the functional characterization of the remaining anti-VWF MAB tested is the subject of another report [28], it is interesting to note that all of the anti-VWF MAB which have been found to inhibit VWF function using a variety of specific functional assays (including ristocetin and botrocetin agglutination procedures) were also capable of inhibiting the PFA-100™ CT.

Antibody inhibition studies have also been undertaken by Kundu et al. [16]. These workers could show inhibition of PFA-100™ CT using antibodies directed to (i) gpIIb/IIIa (at the VWF binding site, MAB AP2), (ii) gp Ib (at the VWF binding site, MAB 6D1), and (iii) VWF (at the gpIIb/IIIa binding site, MAB 9; or at the gp Ib binding site, MAB 202; or at the collagen binding site, MAB 322). The RGDS peptide was also inhibitory to CT, while a polyclonal to fibrinogen was not.

Differences in the PFA-100™ CT Using C/ADP Versus C/Epi Cartridges

Overall, it was evident that the C/Epi cartridge showed the greatest sensitivity to hemostatic disturbances, including those unrelated to VWD. Thus, the C/Epi cartridge showed the least specificity for VWD and greater effects were more persistently observed (i.e., CT more prolonged) in the case of thrombocytopaenia, due to aspirin ingestion, and in the antibody inhibition studies (including an effect using antisera to fibronectin). It is unclear if this anti-fibronectin effect is "real" or if it is an "artifact" reflecting either (i) the greater sensitivity of the C/Epi cartridge to "hemostatic changes" or (ii) some "contaminating" anti-VWF activity in a heterogeneous

polyclonal antibody preparation. Finally, the C/Epi cartridge also yields a wider normal reference range. Some patient samples gave marginally abnormal CT values in our study using the C/Epi cartridge and our own generated reference range but which would be considered normal using the product insert reference range.

CONCLUSIONS AND RECOMMENDATIONS

While an abnormal PFA-100™ CT result should always be followed up, and the presence of VWD should always be confirmed using specific laboratory assays, and utilizing clinical and family history as appropriate [6,7], the PFA-100™ does appear to offer a useful VWD screening process. The PFA-100™ is highly sensitive to the presence of VWD. Notably, while normal PFA-100™ CTs may not exclude a mild Type 1 VWD [32], and will not exclude Type 2N VWD [32], all other VWD samples tested and reported to date [this report; 32,35] have provided abnormal PFA-100™ CTs. Thus, the PFA-100™ may have a role in negative prediction and a normal PFA-100™ CT for both the C/ADP and C/Epi cartridges can be used with some confidence to exclude a severe VWD-like defect. However, since abnormal PFA-100™ CT results can result for a variety of reasons other than VWD (e.g., aspirin, thrombocytopaenia, platelet disorders, low haematocrit), the PFA-100™ has no real specificity for VWD. If the PFA-100™ CT result is abnormal, and one can exclude aspirin, thrombocytopaenia or low haematocrit, then the likelihood of VWD should always be followed up with specific VWF assays. If the PFA-100™ CT result is normal for the C/ADP cartridge, and abnormal for the C/Epi cartridge, then aspirin-like medication may be a possible cause. Because of the cost of the PFA-100™ test cartridges, and the higher sensitivity of the C/Epi cartridge to non-VWD related hemostatic changes, it is probably best to use the C/ADP as the primary VWD screening cartridge, should cost be a major concern. If screening for VWD using the PFA-100™, then performance of a "stat" FVIII:C is also advisable. This will contribute to an assessment of VWD "severity" (particularly for Type 1, Type 2A, Type 2B and Type 3 VWD), as well as assessing the likelihood of Type 2N VWD, or potentially hemophilia A, and the need for further laboratory testing. An overall recommended approach to this process is shown as an algorithm in Fig. 5.

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